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Effect of Genotypes on the Quantification of Hepatitis C Virus (HCV) RNA in Clinical Samples Using the Amplicor HCV Monitor Test and the Quantiplex HCV RNA 2.0 Assay (bDNA)

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The Amplicor HCV Monitor test and the Quantiplex HCV RNA 2.0 (bDNA) assay are two commercially available assays for the quantification of hepatitis C virus (HCV) RNA in clinical samples. A direct comparison of the two assays was carried out using sera frozen previously from patients known to be chronically infected with HCV. Overall, 61 samples from 51 patients were tested simultaneously by the two methods: 67% (28/42) of the patients were infected by HCV genotype/serotype 1, 10 % (4/42) with type 2, and 24% (10/42) with type 3. When the absolute value from each assay was examined, the Quantiplex assay gave a consistently higher reading and the mean logarithmic difference between the two assays was 1.4 (1.0 in type 1, 2.0 in type 2, and 2.2 in type 3). When analyzed according to genotype, strong correlation was observed between the two assays for type 1 ($r = 0.83$, 95% CI 0.63–0.93, $P < 0.01$), but not for nontype 1 samples. Despite the difference in absolute level reported by the two assays, there was a consistent trend of change in HCV RNA concentration by both assays in patients whose consecutive samples were analyzed and the differences between the two assays in consecutive samples were within 0.4 log of each other. The results suggested that with samples containing genotype 1, the Amplicor assay was more sensitive than the Quantiplex assay by about one log. However, the sensitivities of the two assays with nontype 1 samples were much closer probably due to the failure of the Amplicor assay to quantify nontype 1 genotypes effectively. ***J. Med. Virol. 55:191–196, 1998.***

KEY WORDS: HCV RNA concentration; Roche; Chiron; HCV genotype 1 and nontype 1

INTRODUCTION

Hepatitis C virus (HCV) is an important cause of chronic hepatitis for which interferon α is recommended as the treatment of choice. However, the sustained response rate to interferon therapy is only 10–30% [Poynard et al., 1996]. Combination therapy with ribavirin has been tried and preliminary results suggest that this may improve the outcome [Chemello et al., 1995; Schvarcz et al., 1995]. However, a number of factors are known to affect the response rate, including the absence of cirrhosis, the HCV genotype, and the pretreatment level of viremia [Tsubota et al., 1994]. Selection of patients with the best prognostic markers for treatment may therefore improve the chance of a sustained response.

The ability to quantify HCV RNA accurately is important as the pretreatment level may predict response [Lau et al., 1993; Tsubota et al., 1994]. It is also a useful tool for monitoring treatment response and relapse after stopping treatment [Bresters et al., 1994; Gretch et al., 1995]. Two commercial kits, the Amplicor HCV Monitor test (Roche, Switzerland) and the Quantiplex HCV RNA branched DNA assay version 2.0 (Chiron, Emeryville, CA), are currently available for measurement of HCV RNA concentration in serum or plasma. An earlier study compared the Quantiplex 2.0 assay with the Amplicor assay and found that the two assays were comparable in sensitivity and both assays were capable of detecting HCV RNA in patients infected with genotype 2 or 3 [Jacob et al., 1997]. However, another study using plasma from blood donors has suggested that the Amplicor assay did not quantify all genotypes equally, while the Quantiplex version 2.0

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assay gave equal quantification [Hawkins et al., 1997]. In this study, two separate centers in the U.K. (Liverpool and Nottingham) evaluated these two commercial kits using clinical serum specimens, and the combined results with the two assays in a clinical context are reported.

PATIENTS AND METHODS

Patients and Samples

Two centers (Department of Medical Microbiology, University of Liverpool, and Department of Microbiology and Infectious Disease, University of Nottingham) were involved in the comparative study of the two commercial kits. Clinical serum samples from patients with confirmed chronic hepatitis C infection who were being considered for or undergoing interferon therapy were used in the evaluation. The Liverpool center tested 33 samples from 23 patients, while the Nottingham center tested 28 samples from 28 patients. The HCV genotypes or serotypes were previously determined on the test samples or from a previous sample from the same patient. The comparative data between the two centers on the age, HCV genotype/serotype, and presumed source of infection are shown in Table I.

Amplicor HCV Monitor Test

The Amplicor HCV Monitor test is a nucleic acid amplification test for the quantification of HCV RNA in plasma or serum. The measurement of HCV viral RNA is carried out using a quantitation standard (QS), which is a noninfectious RNA transcript that contains the identical primer binding sites as the HCV target and a unique probe binding region that allows the QS amplicon to be distinguished from the HCV amplicon. Reverse transcription and amplification of the conserved 5′ noncoding region target of HCV genomic RNA and QS RNA occurs simultaneously. The protocol recommended by the manufacturer was followed throughout the procedure. Briefly, HCV RNA was isolated from 100 μl of serum by lysis of virus particles with the supplied lysis reagents and the RNA was precipitated with alcohol. A known number of QS RNA molecules was introduced into each specimen with the lysis reagent and carried through the specimen preparation, amplification, and detection steps. A single-step combined reverse transcription and polymerase chain reaction (PCR) was carried out on 50 μl of the prepared specimen (equivalent of 5 μl of serum) using biotin-labeled primers and the enzyme Thermus thermophilus DNA polymerase (rTth pol) in a thermocycler (Perkin-Elmer 2400, Norwalk, CT) with the program recommended by the manufacturer. Serial dilutions of the resultant HCV and QS amplicon were detected using the supplied microtiter plates coated with probes specific to the two amplicons. Color development was effected through the use of avidin–horseradish peroxidase conjugate and 3,3′,5,5′-tetramethylbenzidine substrate. Optical density (OD) was measured at 450 nm. The wells with the lowest OD reading between 0.20 and 2.0 were selected and the dilutions noted. The total OD of HCV test and QS were calculated by multiplying the background-corrected OD reading with the dilution factor associated with the well. The concentration of HCV RNA was calculated using the following formula:

$$(\text{total HCV OD}/\text{total QS OD}) \times (\text{input QS copies/PCR}) \times 200$$

and the result was expressed in HCV RNA copy/ml of serum. The detection limit of the assay claimed by the manufacturer was 2,000 copy/ml.

Quantiplex HCV RNA 2.0 Assay (bDNA)

The Quantiplex HCV RNA 2.0 assay (bDNA) is a signal-amplification nucleic acid probe assay for the quantification of HCV RNA in serum or plasma. The highly conserved 5′ noncoding region and part of the core region of the HCV genome were used as the target for detection. HCV RNA is captured to a well coated with specific synthetic oligonucleotide target probes. A second set of target probes then hybridize to the viral RNA. Branched DNA amplifiers and alkaline–phosphatase-conjugated probes then amplify the signal, which is recorded as luminescent counts by a luminometer using the chemiluminescent substrate Lumi-Phos 530. The quantity of HCV RNA in the sample is determined from a standard curve, which is drawn from the four sets of controls running simultaneously with the samples using the supplied Quantiplex Data Management Software. The protocol recommended by the manufacturer was followed throughout the procedure. Each serum sample was tested in duplicate (50 μl per assay) and the concentration of HCV RNA was calculated as the mean. The sensitivity limit of the version 2.0 assay claimed by the manufacturer was 200,000 equivalence per ml.

Simultaneous Testing of Amplicor and Quantiplex Assay

In order to compare the two tests directly, assays were carried out simultaneously. An aliquot of each sample stored at below −70°C was thawed and prepared for the Amplicor HCV Monitor test. The extracted RNA was stored at −20°C overnight according to instruction. Reverse transcription, amplification, and detection were undertaken the next day. The Quantiplex assay was set up simultaneously with the Amplicor preparation step using the same aliquot of serum and the samples were hybridized on the supplied microtiter plate at 53°C overnight. The rest of the Quantiplex assay was completed the next day.

Genotype and Serotype Determination

Different methods of HCV typing were used by the two centers. The InnoLipa HCV genotyping method (Innogenetics, Belgium) was used in Liverpool, whereas the Murex serotyping ELISA (Murex, U.K.) was used in Nottingham. Since other studies have confirmed that serotypes correspond to genotypes [Simmonds et al., 1993; Bhattacherjee et al., 1995], for the

TABLE I. Differences in Demographic Data and Pretreatment HCV RNA Levels Between the Two Centers

	Liverpool	Nottingham	Total	*P*
Number of samples (patients)	33 (23)	28 (28)	61 (51)	
Male:female ratio	2.8:1	1:1	1.55:1	>0.05[a]
Mean age (range)	38 (29–67)	42 (14–72)	40 (14–72)	>0.2[b]
Genotype/serotype				
1	14	14	28	>0.5[a]
2	3	1	4	
3	6	4	10	
4	0	2	2	
Mixed (1 and 2)	0	1	1	
Not done	0	6	6	
Presumed source of infection				
Blood and blood products	8	13	21	>0.05[a]
Intravenous drug abuse	8	3	11	
Unknown	7	12	19	
Mean pretreatment HCV RNA level (log concentration)				
Amplicor	4.9	5.1	5.0	>0.2[b]
Quantiplex	6.3	6.6	6.5	>0.2[b]

[a]Chi-squared test.
[b]T-test.

purpose of this study, the genotype results from Liverpool, excluding the subtyping information, were analyzed together with the serotype results from Nottingham as if they were identical.

Statistical Analysis

Logarithmic transformation of the readings from the two assays was carried out and parametric statistical methods were employed throughout. Data from the two centers were compared using the t-test or the chi-squared test as appropriate. XY scatter diagrams were drawn and correlation coefficients (r) were calculated for genotype 1 and nongenotype 1 samples that had quantifiable results in both assays using the statistical function of the software Microsoft Excel. With cases from Liverpool where there was more than one sample per patient, only the sample that was taken while the patient was not on interferon therapy was included to avoid repeat analysis of samples from the same patient [Greenhalgh, 1997]. Ninety-five percent confidence intervals (CI) were calculated using the Fisher's z-transformation method [Bland, 1995]. An equation relating the log Amplicor results to log Quantiplex results was calculated with genotype 1 samples using the linear regression statistical functions in Excel. To analyze consecutive samples from the same patient, the logarithmic changes between samples by the two assays were compared.

RESULTS

A total of 61 samples from 51 patients were studied. Serial samples from patients were included in the Liverpool center but all samples tested in Nottingham were from separate patients. The overall male-to-female ratio was 1.55 and the mean age was 40. All patients from Liverpool were typed as either type 1, 2, or 3. Six patients from Nottingham did not have a serotype result; two patients had serotype 4 infection; and one patient had a mixed type 1 and 2 infection. Overall, 67% (28/42) of the patients were infected by type 1, 10% (4/42) by type 2, and 24% (10/42) by type 3. Twenty-one patients (41%) acquired HCV infection through receiving contaminated blood or blood products, mainly factor VIII; 11 patients (22%) probably acquired HCV infection through intravenous drug abuse, but a large proportion (37%) did not have known risk factors. There was no significant differences between the two centers with regard to sex ratio, age, and genotype/serotype distribution. Ten samples from Liverpool were taken during interferon therapy, whereas all 28 samples from Nottingham were taken before treatment. There was no significant difference in the pretreatment HCV RNA concentration from both centers using either assay (Table I).

With the Amplicor assay, 14 samples had HCV RNA below the detection limit. The range of detectable HCV RNA concentration expressed in logarithm was from 3.4 to 6.0, with a median log concentration of 5.4. With the Quantiplex assay, 13 samples had HCV RNA below the detection limit. The range of detectable HCV RNA concentration expressed in logarithm was from 5.4 to 7.8, with a median log concentration of 6.5. When samples with detectable HCV RNA concentration by both assays were analyzed, the absolute readings from the Quantiplex assay were higher than that of the Amplicor assay by a mean of 1.4 log. When analyzed according to genotypes, the Quantiplex assay gave a higher reading by 1.0, 2.0, and 2.2 log, respectively, for types 1, 2, and 3. Six samples (9.7%) had discrepant Amplicor and Quantiplex results on HCV RNA detection: three samples had HCV RNA detected by Amplicor but not by Quantiplex (two type 1 and one type 3) and three had HCV RNA detected by Quantiplex but not by Amplicor (one type 1 and two type 3). Four of these six discrepant samples had a low concentration of HCV RNA within 0.4 log above the detection limit of the detecting assay.

When analyzed according to genotype (excluding un-

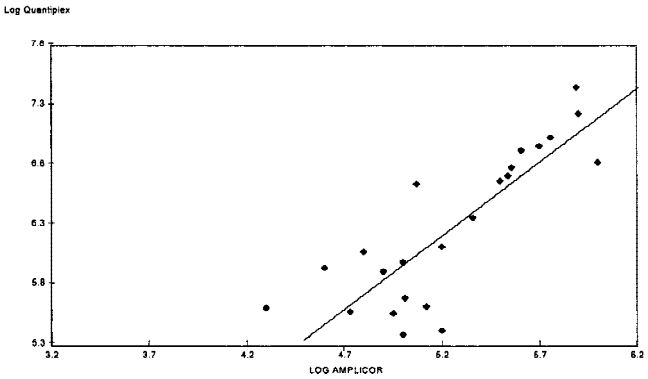

Fig. 1. Log Amplicor vs. log Quantiplex results for HCV type 1 samples only.

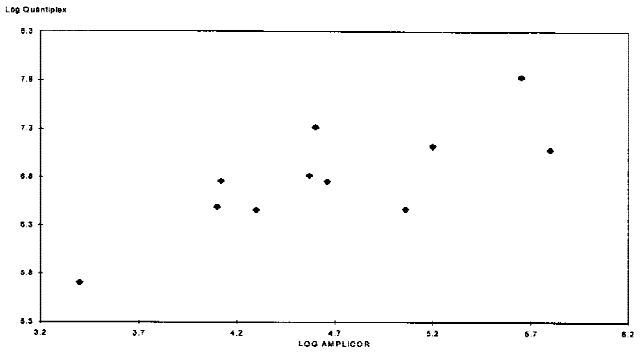

Fig. 2. Log Amplicor vs. log Quantiplex results for nontype 1 samples.

typed samples and mixed-type infection), a strong and significant correlation was observed between the two assays (Fig. 1) with type 1 samples (r = 0.83, 95% CI 0.63–0.91, $P < 0.01$), but not with nontype 1 (Fig. 2). Regression was used to relate Amplicor results to Quantiplex results with genotype 1 samples, and the resultant regression line was represented by the equation

$$\log [\text{Quantiplex}] = 1.17267 \times \log [\text{Amplicor}] + 0.1087.$$

There was no significant correlation of genotype 2 HCV RNA concentration measured by the two assays ($P > 0.05$). The correlation of genotype 3 HCV RNA concentration between the two assays had a wide confidence interval (0.03–0.99, $0.05 > P > 0.01$). The number of genotype 2 and 3 samples with quantifiable results in the series was much smaller than that of genotype 1, affecting the power of the analysis.

Ten patients from Liverpool each had two consecutive samples included in the analysis. These specimens were collected at an interval between 3–12 months. The logarithmic changes between the samples as recorded by both assays were analyzed (Table II). Excluding patient 5, who had a marked discrepancy between the two assays, all the other paired samples had similar trends of changes and the variation between the two assays was within 0.4 log.

TABLE II. Logarithmic Changes in HCV RNA Concentration in Patients With Consecutive Samples Measured by the Amplicor Assay and the Quantiplex Assay*

		Log changes in concentration	
Patients	Genotype	Amplicor	Quantiplex
1	1a	5.9–<3.2	7.2–<5.3
2	1a	5.2–5.5	6.1–6.1
3	1a	<3.2–<3.2	<5.3–<5.3
4	1b	5.1–4.6	6.8–6.5
5[a]	1b	6.0–<3.2	6.8–6.6
6	2a	5.8–4.8	7.1–5.8
7	2b	3.4–3.9	5.7–6.4
8	2b	<3.2–5.2	<5.3–7.1
9	3a	3.4–<3.2	<5.3–<5.3
10	3a	<3.2–<3.2	<5.3–5.7

*<3.2 and <5.3 indicate results below the detection limit of Amplicor and Quantiplex assays, respectively.
[a]The second sample had a markedly discrepant result between the Quantiplex assay and the Amplicor assay.

DISCUSSION

Although the two centers adopted the same strategy for comparing the two HCV RNA quantification assays, the profiles of patients that were chosen were slightly different. The Nottingham samples were from separate patients, whereas repeat samples from the same patients were included in Liverpool. There was, however, no significant difference between the two centers with regards to patient demography and the HCV RNA concentration of all pretreatment samples were comparable by both assays.

The absolute readings as reported by the Amplicor assay were lower than those of the Quantiplex assay, with a mean logarithmic difference of 1.4. The earlier version of the Quantiplex assay (version 1.0) has been reported to correlate well with a number of in-house competitive reverse transcription PCR assays [Bresters et al., 1994; Gretch et al., 1995; Mayerat et al., 1996; Toyoda et al., 1996]. When version 1.0 was compared to the Amplicor assay, a positive correlation between the two assays was found, but the results could not be used interchangeably because of systematic differences [Hadziyannis et al., 1997]. Another study that compared an in-house quantitative PCR assay with the Amplicor assay found a significant lower reading with the Amplicor assay when the sera contained more than 5×10^5 copy/ml [Roth et al., 1996]. A previous direct comparison of the Quantiplex 2.0 assay with the Amplicor assay using samples from blood donors suggested that the Amplicor assay, unlike the Quantiplex 2.0 assay, did not quantify all genotypes equally [Hawkins et al., 1997]. However, another study found the two assays comparable in sensitivity and capable of detecting all common genotypes [Jacob et al., 1997]. It is important to point out, however, that the two assays are based on different principles and use different quantification standards of different natures, lengths, and sequences [Pawlotsky, 1997]. It is therefore not possible to compare the absolute concentrations calcu-

lated by the two methods. A similar situation has been observed with analysis of HIV viral load using different methodologies [Schuurman et al., 1996]. A correlation analysis of the two assays is therefore more appropriate. When the two assays were examined according to genotype, the two assays had a good correlation for genotype 1 samples but not for nongenotype 1 samples.

The previous version of the Quantiplex assay (version 1.0) was criticized for the lack of sensitivity for genotypes other than type 1. In order to achieve equal quantification of all three genotypes, correction factors had to be introduced for genotypes 2 and 3 [Lau et al., 1995]. This problem appears to have been solved in the version 2.0 assay and there is evidence that the version 2.0 assay has equal sensitivity in quantification of the three main genotypes [Lau et al., 1995; Detemer et al., 1996]. The Amplicor assay, however, makes no claims about genotype. The primers used in the Amplicor assay are designed to amplify HCV genotype 1, and have mismatches to genotypes 2 and 3 [Smith et al., 1995]. These primers may not amplify genotype 2 and 3 targets efficiently in the presence of the competitive QS that matches the primers perfectly. The lack of a consistent correlation between the two assays on nontype 1 samples and the finding that the mean value of nontype 1 samples was at least 2 log below the Quantiplex reading both support the previous finding of unequal quantification of genotypes by the Amplicor assay [Hawkins et al., 1997]. A similar quantitative assay for human immunodeficiency virus (HIV), the Amplicor HIV monitor assay, is known to underestimate HIV RNA concentration in patients who were infected with non-B, -C, and -D subtypes [Coste et al., 1996]; this problem is solved by introducing primers that match the variant subtypes [Michael et al., 1997].

Considering the respective detection limits given by the manufacturers, which is 2 log lower for the Amplicor than for the Quantiplex assay, our results suggested that the sensitivity of the two assays were much closer than they appeared to be. With genotype 1 samples, the Amplicor assay is probably one log more sensitive than the Quantiplex assay; but their sensitivities are similar with samples containing genotypes 2 and 3. Overall agreement of the two tests for qualitative HCV RNA detection was 90% (56/62). Four of the six samples with discrepant HCV RNA detection results had HCV RNA concentrations very close to the detection limit of the detecting assay (within 0.4 log). There was one sample that showed a marked discrepancy between the two assays (patient 5, Table II). Although it was not possible to determine whether this was due to an Amplicor assay false negative result or a Quantiplex assay false positive result, the clinical state of the patient, as well as analysis of previous and subsequent samples from this patient, suggested the former explanation. Of the other five discrepancies, the two genotype 1 samples with detectable RNA by Amplicor but not by Quantiplex was probably the result of a higher sensitivity of the Amplicor assay with genotype 1. The failure of the Amplicor assay to detect RNA in the two genotype 3 samples positive by Quantiplex could be due to the defect of the Amplicor assay to detect non-1 genotype. The remaining discrepant sample, which contained genotype 3, had a low level of RNA (2,254 copy/ml by the Amplicor assay) and should be considered as a borderline sample for both assays. When the logarithmic changes in HCV RNA concentration between longitudinal samples were studied (excluding patient 5), the differences between the Quantiplex assay and the Amplicor assay was within 0.4 log with all three genotypes (Table II). This suggested that no matter what level of HCV RNA concentration is reported by the two assays, the recorded changes between samples remain more or less consistent with both assays.

PCR-based assays are generally expected to be more sensitive and have a lower limit of quantification compared to hybridization-based assays [Bresters et al., 1994; Gretch et al., 1995]. However, despite the difference in their claimed detection limit, we found that the sensitivity of the two assays were much closer than they appeared to be. The values as determined by the two assays are not directly comparable to each other. Results from the two methods, however, had a good correlation when genotype 1 samples were tested and the results can be converted from one to another using a simple equation. Unlike the Quantiplex assay, the Amplicor assay did not seem to quantify HCV genotypes 2 and 3 as effectively as genotype 1; this may cause problem if the assay is used to determine pretreatment viral load as a guide to prognosis. Because of the nature of our study, clinical correlates were not available and we cannot address the question of the relative relevance of the two assays for therapeutic indication and monitoring. Nevertheless, for the purpose of monitoring, the change in level with time is more important than the absolute level. From the results reported herein, both assays detected similar changes in viral load regardless of genotype. Thus, both assays can be used for monitoring HCV RNA level in response to therapy.

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